Tetrapeptide regulating blood glucose level in diabetes mellitus

The invention refers to the field of medicine and may be applied for the diabetes mellitus treatment as a substance regulating glucose concentration.

Among the closest indication analogues of this substance, there are known insulin preparations, used for type 1 diabetes mellitus treatment. The choice preparation is a recombinant or genetically modified short acting human insulin preparation: Actrapid, Humulin R, Insuman R, Biosulin R, and human insulin preparations of the prolonged effect: Protophane, Humulin N, Insuman-Basal and Biosulin N - human insulin analogues of the short-term and prolonged effect. Preparations Humalog and Novorapid, are the preparations of ultra-short effect group, they are notable for the quick onset effect and a little bit short period of peak effect compared to the insulins of the short effect; they are effective in diminishing decrease of postprandial glucose level. Nevertheless insulin therapy is a replacing therapy, which can lead to some complications, such as allergic reactions, hypoglycemic states, insulin resistance and post-insulin lipo-dystrophy. The type 2 diabetes mellitus medical therapy has also changed due to the introduction into the clinical practice of short-term effect preparations, such as Novonorm or Repaglinid and Starlix. There are traditionally used sulphonylureas, such as: Glibenclamide group, Glipizide, Gliklazide group, Glikvidon and Amaril (Glimepirid), which is a prolonged effect preparation and has significant benefits compared to other preparations of this group. Diabeton MR and Glibenese-retard are preparations of the prolonged effect. It should be noted, that patients suffering diabetes mellitus often reveal, so-called, secondary resistance towards sulphonylureas, this is caused by the elevation of residual insulin secretion. Sulphonilureas have significant side effects: dyspeptic disorder, allergic reactions, bone marrow function oppression, toxic effect on liver and kidney, hypoglycemia. There are also used biguanids, such as metformin (Glucophage, Siofor e.t.c.), Glitazones or insulin sensitizers: Actose and Avandia and alpha-glycosidase inhibitors: Acarbose and Meglitol. These oral preparations increase tissue sensitivity to insulin and exert positive normalising effect on carbohydrate metabolism. Nevertheless their application can be restricted due to low effectiveness or side effects (Balabolkin M. I., Diabetology. – M.: Meditzina, 2000. – 672 p.: Register of Pharmaceutical Substances of Russia. Edition 10. – RLS-2003, Moscow. – 2003. – 1438 pp.)

There is known a decapeptide insulin fragment (Patent of the Russian Federation No.2078769 «Peptide fragment, possessing biological insulin similar activity», International Classification of Inventions (ICI) A61 K 38/28, 1997), revealing biological activity similar to that of insulin.

There are known the peptide p277 (epitop of the human heat shock protein (hsp 60) analogues (Patent of the Russian Federation No.2159250 «Peptide p277 analogues and

pharmacological substances on its basis for treatment and diagnostics of diabetes mellitus», International Classification of Inventions (ICI) A61 K 39/00,38/00, 2000).

Nevertheless, the biological activity, described in the above patents, reveals in the insulin-like effect of these peptides and can be used in order to invent peptide substances for type1 diabetes mellitus treatment.

There are known small insulin-potentiating peptides, described in the patent (EP № 1268518 «Insulin potentiating peptides», International Classification of Inventions (ICI) C07K5/10; A61K38/07; A61K38/08, 2001), which was taken as a prototype for the pharmaceutical preparation and method of prevention or/and treatment for diabetes mellitus. These small peptides can serve as peptide pharmaceutical agents, which can be used in treatment for diabetes mellitus.

It should be noted that the proposed peptide compound is a tetrapeptide, which has no structural analogues.

The proposed invention is designed to obtain a new biologically active compound of peptide nature regulating the glucose level in the patients suffering type 2 and type 1 diabetes mellitus.

The present invention describes a new tetrapeptide lysyl-glutamyl-aspartyl-tryptophane amid of the general formula Lys-Glu-Asp-Trp-NH_2 of sequence 1 [SEQ ID NO:1].

The tetrapeptide is obtained by a classical method of peptide synthesis in a solution (Yakubke Kh.-D., Eshkeit Kh. Amino acids, peptides, proteins: Translated from German. – Mir, Moscow. – 1985. – 456 pp.).

The present invention describes tetrapeptide lysyl-glutamyl-aspartyl-tryptophane amid of the general formula Lys-Glu-Asp-Trp-NH_2 of sequence 1 [SEQ ID NO:1] revealing biological activity, and namely, regulating glucose level.

The regulatory effect of Lys-Glu-Asp-Trp-NH_2 tetrapeptide on the blood glucose level has been revealed experimentally in alloxan diabetes. It is believed that alloxan diabetes is associated with the injury of the β-cells of the pancreas and is accompanied by the pronounced hyper glycaemia due to insulin deficiency and glyconeogenesis activation.

The tetrapeptide Lys-Glu-Asp-Trp-NH_2 was experimentally proved to be non-toxic.

The pharmaceutical substance of the present invention contains as its active peptide agent an effective amount of tetrapeptide lysyl-glutamyl-aspartyl-tryptophane amid of the general formula Lys-Glu-Asp-Trp-NH_2 of the sequence 1[SEQ ID NO:1] and regulates glucose level in case of diabetes mellitus.

The notion "pharmaceutical substance" under this application implies the use of any drug form containing the effective amount of the tetrapeptide of the general formula Lys-Glu-Asp-Trp-NH_2, which can find its preventive and/or therapeutic employment in medicine as a substance regulating blood glucose level in case of diabetes mellitus.

The notion "therapeutically effective amount" under this application implies the use of such an amount of the active peptide agent, which, in compliance with the quantitative indices of its activity and toxicity, as well as with respect to the special knowledge available, shall be effective in this drug form.

To obtain pharmaceutical compositions meeting the invention, the proposed tetrapeptide is blended as an active ingredient with a pharmaceutical carrier in accordance with the methods of compounding accepted in pharmaceutics.

The carrier may have various forms depending on the drug form of the substance desirable for introduction into a body, for example parenteral or oral administration.

To produce drug compositions of desirable dosed form for oral administration there can be used all known pharmaceutical components.

The carrier for parenteral administration usually includes sterile water, though there could be employed other ingredients instrumental for stability or maintaining sterility.

The proposed invention presupposes that the pharmaceutical substance should be preferably prescribed for the parenteral or oral administration.

The proposed invention also refers to the method of prevention and/or treatment of diabetes mellitus, which consists in administering to the patient of the pharmacological substance, containing as an active peptide agent an effective amount of tetrapeptide Lys-Glu-Asp-Trp-NH_2 in doses of 0.1 –30 μg/kg of the body weight at least once a day during the period necessary to obtain therapeutic effect.

The method of prophylaxis and/or treatment of diabetes mellitus consists in preventive or treatment parenteral or oral administering to the patient of the pharmacological substance.

The proposed tetrapeptide is active when introduced in doses of 0,1-30 μg/kg of the body weight, though lower/higher doses are admissible depending on the character and severity of the treated pathologic process.

Technical result of the proposed invention is a regulation of the glucose level due to the insulin secretion increase and increase of tissue sensitivity to insulin.

The possibility of obtaining an objective technical result of the invention application is affirmed by the reliable experimental and clinical data obtained by the methods established in this field of science.

The invention is illustrated by the tables.

Table 1 demonstrates the effect of tetrapeptide Lys-Glu-Asp-Trp-NH_2 on blood glucose level of the rats with alloxan diabetes (treatment).

Table 2 demonstrates the effect of tetrapeptide Lys-Glu-Asp-Trp-NH_2 on blood glucose level of the rats with alloxan diabetes (prevention and treatment).

Table 3 demonstrates the effect of tetrapeptide Lys-Glu-Asp-Trp-NH_2 in different doses on blood glucose level of the rats with alloxan diabetes.

Table 4 demonstrates the effect of tetrapeptide Lys-Glu-Asp-Trp-NH_2 on insulin level in blood of the rats with alloxan diabetes.

Table 5 demonstrates the results of the glucose tolerance test in the rats with aloxan diabetes (the 44^{th} day after the tetrapeptide Lys-Glu-Asp-Trp-NH_2 course completion).

Table 6 demonstrates insulin effect on blood glucose level of the rats with alloxan diabetes (the 28^{th} day after the tetrapeptide Lys-Glu-Asp-Trp-NH_2 course completion).

Table 7 represents distribution of the patients with diabetes mellitus during the study.

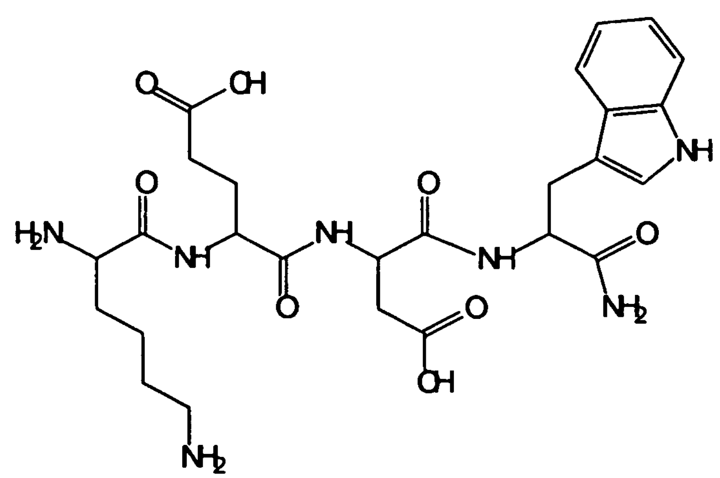
Table 8 demonstrates the effectiveness of the tetrapeptide Lys-Glu-Asp-Trp-NH_2 parenteral administration to patients, suffering type 1 and type 2 diabetes mellitus, who were treated with insulin.

The proposed invention is illustrated by the example of the tetrapeptide Lys-Glu-Asp-Trp-NH_2 synthesis (Example1), by the examples of the tetrapeptide Lys-Glu-Asp-Trp-NH_2 biological activity (examples 2, 3, 4, 5, 6, 7), and by the example of the results of tetrapeptide Lys-Glu-Asp-Trp-NH_2 clinical application, which demonstrates its pharmacological properties and confirms the possibility to achieve prophylactic or/and treatment effect (example 8).

Example 1. Synthesis of Lys-Glu-Asp-Trp-NH_2 tetrapeptide

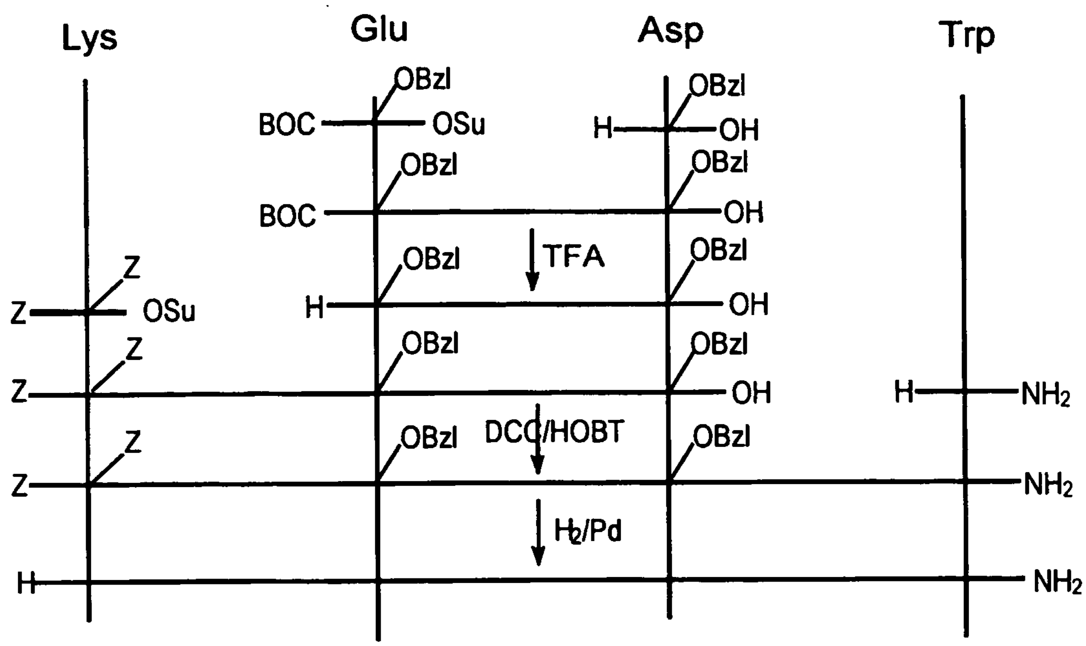
1. Product name: **lysyl-glutamyl-aspartyl-tryptophane amid**
2. Structural formula:

H-Lys-Glu-Asp-Trp-NH_2



3. Molecular formula without ion pair: $C_{26}H_{37}N_7O_8$.
4. Molecular weight without ion pair: 575,62.
5. Ion pair: none.
6. Appearance: white amorphous powder without smell.

7. Method of synthesis: the peptide is obtained by a classical method of synthesis in a solution by the following scheme:



Z – benzyloxycarbonyl group;
BOC – tert.butyloxycarbonyl group;
OSu – N-oxysuccinimide ester;
OBzl – benzyl ester;
DCC – N,N′-dicyclohexylcarbodiimide;
HOBT – N-oxybenzotriazol.

N,N′-dimethylformamide was used as a solvent. When adding aspartic acid, the defence of α-COOH group was applied by salification with triethylamine. BOC-protecting group was removed with trifluoracetic acid (TFA) solution and Z-protecting groups - with catalytic hydrogenation. The product was extracted and purified by the method of preparative chromatography on a normal phase column (silicagel).

Properties of the finished product:

- amino acid analysis

	Lys	Glu	Asp	Trp
	0.95	**1.00**	**1.06**	**0.90**

- peptide content 97.48 % (by HPLC, 220 nm);
- thin layer chromatography (TLC) – individual, R_f=0.64 (plate PTSX-P-V-UV Sorbfil, silicagel STX-1VE 8-12 μm acetonitrile : water 3:1);
- moisture content: 7 %;
- pH of 0.01 %-solution: 4.05;

- UV-spectrum: the highest point at 280 nm – trypthophane indole ring
- specific rotary power: $[\alpha]_D^{23}$: -26.53° (c=1.0; H_2O), "Polamat A", Carl Zeiss Jena

Example of synthesis:

1. **BOC-Glu(OBzl)-Asp(OBzl)-OH(I), N-tert.butyloxycarbonyl-(γ-benzyl)glutamyl-(β-benzyl)aspartate.**

   4.34 g (0.0100 mol) of N-oxysuccinimide ester of N-tert.butyloxycarbonyl-(γ-benzyl)glutamic acid (BOC-Glu(OBzl)-OSu) is dissolved in 20 ml of dimethylformamide and added 1.72 ml (0.0125 mol) of triethylamine and 2.80 g (0.0125 mol) of β-benzyl aspartate. The mixture is stirred for 24 hours at room temperature. Afterwards the product is precipitated with 0.5N sulphuric acid solution (150 ml), extracted by ethyl acetate (3x30 ml), washed in 0.5N sulphuric acid solution (2x20 ml), water, 5 % sodium bicarbonate solution (1x20 ml), water, 0.5N sulphuric acid solution (2x20 ml), water. The product is dried over anhydrous Na_2SO_4. Ethyl acetate is filtered and removed *in vacuo* at 40°C, the residue is dried *in vacuo* over P_2O_5. 5.68 g (≈100 %) of oil is obtained. R_f=0.42 (benzene-acetone 2:1, Sorbfil plates, Silicagel - 8-12 μm, development by UV and chlorine/benzidine).

2. **TFA·H-Glu(OBzl)-Asp(OBzl)-OH (II), (γ-benzyl)glutamyl-(β-benzyl) aspartate trifluoracetate**

   5.68 g (≈0.01 mol) of N-tert.butyloxycarbonyl-(γ-benzyl)glutamyl-(β-benzyl)aspartate (I) is dissolved in 20 ml of dichlormethan-trifluoracetic acid mixture (3:1). Two hours later the solvent is removed *in vacuo* at 40°C. The removal is repeated with an addition of another portion of dichlormethan (2x10 ml). The residue is dried *in vacuo* over NaOH. 5.80 g (≈100 %) of oil is obtained. R_f=0.63 (n-butanol-pyridine-acetic acid-water, 15:10:3:12).

3. **Z-Lys(Z)-Glu(OBzl)-Asp(OBzl)-OH (III), N,N$^\varepsilon$-dibenzyloxycarbonyllysyl-(γ-benzyl)glutamyl-(β-benzyl)aspartate.**

   5.65 g (0.01 mol) of (γ-benzyl)glutamyl-(β-benzyl)aspartate trifluoracetate (II) is dissolved in 10 ml of dimethylformamide, added 2.80 ml (0.02 mol) of triethylamine and 6.64 g (0.013 mol) of N-oxysuccinimide ester of N,N$^\varepsilon$-dibenzyloxycarbonyllysine. The reacting mixture is stirred for 24 hours at room temperature.

   The product is precipitated with 0.5n sulphuric acid solution (150 ml), extracted by ethyl acetate (3x30 ml), washed in 0.5n sulphuric acid solution (2x20 ml), water, 5 % sodium bicarbonate solution (1x20 ml), water, 0.5n sulphuric acid solution (2x20 ml), water and dried over anhydrous sodium sulphate. Ethyl acetate is filtered and removed *in vacuo* at 40°C. The residue is recrystallised in the ethyl acetate/hexane system. The product is filtered

and dried *in vacuo* over P_2O_5. The yield is 6.04 g (72 %). The temperature of melting (T_{ml}) is 142°C. R_f=0.60 (benzene-acetone, 1:1).

4. **Z-Lys(Z)-Glu(OBzl)-Asp(OBzl)-Pro-OBzl (IV), 1024,15 N,N$^{\varepsilon}$-dibenzyloxycarbonyllysyl-(γ-benzyl)glutamyl-(β-benzyl)aspartyl-tryptophan amid.**

1.8 g (7,2 μmol) of tryptophan amid hydrochloride (HCl·H-Trp-NH_2) is suspended in 15 ml of tetrahydrofuran and added 1.0 ml (7,2 mmol) of triethylamine while stirring. In 5 minutes 4.0 g (4.8 mmol) of N,N$^{\varepsilon}$-dibenzyloxycarbonyllysyl-(γ-benzyl)glutamyl-(β-benzyl)aspartate (III) and 0.8 g (5.8 mmol) of N-oxybenzotriazol are added. The mixture is cooled down to 0°C. Afterwards, 1.2 g (5.8 mmol) of N,N′-dicyclohexylcarbodiimide solution cooled down to 0°C is added in 5 ml of tetrahydrofuran. The mixture is stirred at this temperature for 2 hours and left to blend for a night at room temperature. The reaction mixture is poured into the ice-cold water (150 ml), the residue is grinded and filtered out. The residue is suspended in ethyl acetate (200ml) and the generated gel is washed consecutively 1 N H_2SO in water (2x100 ml), 5% $NaHCO_3$ (2x100 ml), 1 N H2SO_4 (2x100 ml) in water (2x100 ml), in saturate NaCl solution. The solvent is removed *in vacuo* and the product is twice crystallised in the isopropyl alcohol. The yield is 4.9 g (95 %), R_f=0.67 (benzene-acetone, 2:1).

5. **H-Lys-Glu-Asp-Trp-NH_2 (V), lysyl-glutamyl-aspartyl-triptophane amid, 575,62**

4.7 g of N,N$^{\varepsilon}$-dicarbobenzenoxylysyl-(γ-benzyl)glutamyl-(β-benzyl)aspartyl-triptophane amid (IV) is hydrogenated in the methanol/water (5:1) system over Pd/C catalyst. Completeness of the deblocking reaction is monitored by TLC method in the benzene/acetone (2:1) and acetonitrile/water (1:3) systems. At the reaction completion the catalyst is filtered out, the filtrate is removed *in vacuo* and the residue is recrystallised in the water/methanol system. The product is dried *in vacuo* over KOH. The yield is 2.6 g (90 %). R_f=0.64 (acetonitrile/water, 1:1).

For purification, 2,6 mg of the product is dissolved in 5 ml acetontitril-water mixture (1:3) and put it on the column 21x4,5 cm with "Sigma" silicagel, 230-400 bags (40-63 μ). Eluation by the acetonitril-water (1:3) system. There was obtained 1 g chromatographically homogeneous substance.

6. **Analysis of the finished product.**

- Peptide content is defined by HPLC on Nucleosil column C18 4,6x250 mm. A: 0,1% TFA; B: MeCN; grad. B 0→30% in 30 min. The flow speed equals 1 ml/min. Detection by 220 nm, scanning - by 190-600 nm, the sample volume is 20 μl. Peptide content - 97.48 %.
- Amino acid analysis is carried out on AAA "T-339" tester, Prague.

Lys	Glu	Asp	Trp
0.95	1.00	1.06	0.90

- TLC: individual, R_f=0.64 (acetonitrile/water, 3:1, Sorbfil plates, 8-12 μm Silicagel, developing in chlorine/benzidine and UV).
- Moisture content: 7 % (gravimetrically, according to the mass loss by drying, - 20 mg at 100°C).
- pH of 0.01 % solution: 4.05 (potentiometrically)
- Specific rotary power: $[\alpha]_D^{23}$: -26.53° (c=1.0 H_2O), "Polamat A", Carl Zeiβ Jena.
- UV-spectrum: peak by 280 nm – indole ring of triptophane, "Beckman DU 650", 0,001% water solution.

Example 2. Effect of tetrapeptide Lys-Glu-Asp-Trp-NH_2 on the course of alloxan diabetes in rats (treatment variant)

The study was conducted on 21 white mongrel male rats with average body weight 375±35 g. After estimation of glucose concentration in the blood all the animals were divided at random into 2 groups. Then all the animals were exposed to single intravenous administration 1 ml of alloxan solution ("Spofa") in dose 35 mg/kg. In 15 days control animals were administered once a day intraperitoneally with 0,3 ml 0,9% NaCl solution, rats of the main group were administered with Lys-Glu-Asp-Trp-NH_2 in dose 3 μg (in 0,3 ml of 0,9% NaCl solution) per rat during 11 days.

Table 1 shows the results of the study which reveal that Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration contributed to the reliable decrease of the blood glucose level in the animals throughout the whole study by 38,4% (30-47,7%). Lys-Glu-Asp-Trp-NH_2 tetrapeptide -related glucose level decrease correlated with the lethality decrease in animals of the main group. So in the animals of the control group by the end of the investigation (84 days after alloxan administration) lethality was 70%, while in the rats which were administered with Lys-Glu-Asp-Trp-NH_2 tetrapeptide – 36,4%. Thus the Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration enabled two-fold lethality decrease in alloxan diabetes animals.

Example 3. Effect of Lys-Glu-Asp-Trp-NH_2 on the course of alloxan diabetes in rats (prophylaxis and treatment variant)

The experiment was carried out on 15 white mongrel male rats with the average body weight 375±35 g. The animals were divided randomly into 2 groups. Control animals were administered once a day intravenously with 0,3 ml of 0,9% NaCl solution, while the main group animals were administered with Lys-Glu-Asp-Trp-NH_2 tetrapeptide in dose of 3 μg (in 0,3 ml of 0,9% NaCl solution) per rat during 7 days. After that all the animals were subject to single intravenous

administration of 1 ml of alloxan solution ("Spofa") in dose 35 mg/kg. Lys-Glu-Asp-Trp-NH_2 tetrapeptide had been administered during 3 days following alloxan administration. After that control rats were subject to the second Lys-Glu-Asp-Trp-NH_2 tetrapeptide course from 18 day till day 28 (total 11 days) in the same dose.

The results of the study are shown in table 2. First of all, it should be mentioned that Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration to healthy animals did not lead to the decrease of blood glucose level. Control animals during the whole experimental period after alloxan administration revealed diabetes mellitus development accompanied by the increased glucose concentration in the blood 1,9-4,9 times as compared to initial level. The rats subjected to one course of Lys-Glu-Asp-Trp-NH_2 tetrapeptide revealed a decrease of blood glucose level by 22-30% as compared to the controls. After the second course of Lys-Glu-Asp-Trp-NH_2 tetrapeptide these animals revealed a complete normalisation of the blood glucose level in all experimental periods (the 28th, 33rd, 40th day), while in the animals of the control group the blood glucose level was correspondently 2; 4.2;3.8 fold increased.

It should be noted, that only 2 rats out of 8 treated with Lys-Glu-Asp-Trp-NH_2 tetrapeptide reported severe form of diabetes mellitus, while in the control group there were 5 rats out of 7, thus, in the control group this index was 2,9fold higher.

On completion of the study (the 40th day after alloxan administration), there survived 57.1% of the control animals and 75% of the animals treated with Lys-Glu-Asp-Trp-NH_2 tetrapeptide.

The results of the study show that Lys-Glu-Asp-Trp-NH_2 tetrapeptide contributes to the normalisation of the glucose level in alloxan diabetes rats, which is accompanied by the decrease of lethality.

Example 4. Effect of Lys-Glu-Asp-Trp-NH_2 tetrapeptide in different doses on the course of alloxan diabetes in rats

The study was performed on 23 white mongrel male rats weighing on average 375±35 g. All the animals were subject to single intravenous administration of 1 ml of alloxan ("Spofa") solution in dose of 35 μg/kg.

Then the animals were divided at random into 3 groups. Control animals were intraperitoneally administered with 0.3 ml of 0.9% NaCl solution once a day. Rats of the second and third group were administered with tetrapeptide Lys-Glu-Asp-Trp-NH_2 in dose of 1 μg (in 0.1 ml of 0.9% NaCl solution) and 10μg (in 1,0 ml of 0.9% NaCl solution) per rat during 7 days.

Table 3 demonstrates the results of this experiment. The administration of tetrapeptide Lys-Glu-Asp-Trp-NH_2 to rats in dose of 1μg contributed to the pronounced increase in the blood glucose level on the 1st and the 4th days after completion of the tetrapeptide course by 17,3 and

12,3% correspondingly as compared to the controls. Tetrapeptide Lys-Glu-Asp-Trp-NH_2 administration to rats in dose of 10 μg led to even more pronounced decrease of glucose level by 30; 23.8; 26; 12.7% on day 1, 4, 7, 17 correspondingly. These data show that the increase of tetrapeptide Lys-Glu-Asp-Trp-NH_2 dose pronouncedly effects on blood glucose level of animals.

Example 5. Effect of tetrapeptide Lys-Glu-Asp-Trp-NH_2 on blood glucose level of alloxan diabetes rats

The experiment was held on 18 white mongrel male rats weighing on average 375±35 g. After the estimation of the blood glucose level all the animals were divided at random into 2 groups. Then all the animals were subjected to single intravenous injection with 1 ml of alloxan solution ("Spofa") in dose of 35 mg/kg. 15 days later control animals were daily intraperitonealy administered with 0.3 ml of 0.9% NaCl solution, rats of the main group - with tetrapeptide Lys-Glu-Asp-Trp-NH_2 in dose of 3 μg (in 0.3 ml of 0.9% NaCl) per rat during 11 days.

The results of the experiment are represented in table 4, which demonstrates that on day 15 after alloxan administration the animals reported diabetes mellitus. In the rats administered with tetrapeptide Lys-Glu-Asp-Trp-NH_2 insulin content in the blood during 8 days after the substance had been administered was 3,9 fold higher than in rats of the control group. All the following estimations conducted during the experiment revealed some amount of insulin in the blood of the rats (13-18%), though there was no insulin at all in the blood of the control animals. On completion of the experiment (on the 70^{th} day after alloxan administration) 62,5% of the control animals were alive, in the animals administered with tetrapeptide Lys-Glu-Asp-Trp-NH_2 70% were alive.

The analysis of the results of this experiment showed that administration of tetrapeptide Lys-Glu-Asp-Trp-NH_2 to animals with alloxan diabetes contributes to the maintenance of the insulin blood level, which can result from the partial restoration of the insulin producing cell structure and function.

Example 6. Effect of tetrapeptide Lys-Glu-Asp-Trp-NH_2 on indices of sugar curve in alloxan diabetes rats (treatment variant)

The study was conducted on 13 male rats, enrolled in the previous tests (treatment variants – 44 days after completion of tetrapeptide Lys-Glu-Asp-Trp-NH_2 administration). 7 healthy rats with the same body weight constituted a separate group. All the animals were administered intravenously with 1 ml of 2% glucose solution, after that glucose concentration in their blood was estimated.

Table 5 demonstrates the results of the trial, which reveal that in healthy rats after glucose

administration its concentration was 5 min later – 203.9%, 30 min – 156.3%, 60 min – 124.6%, 120 min – 114.5% compared to the initial level (100%). In control animals the same index was correspondingly 129.8; 127.5; 123.5; 121.1%. These data point at the strong suppression of the pancreas function after alloxan lesion. The same index in rats, which were administered with Lys-Glu-Asp-Trp-NH_2 tetrapeptide was 142.9; 97.3; 95.6; 77.9%. The results of this trial reveal that Lys-Glu-Asp-Trp-NH_2 tetrapeptide can stimulate pancreas function in rats, suffering alloxan diabetes.

Example 7. Insulin effect on blood glucose level in alloxan diabetes rats after Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration.

The study was conducted on 13 male rats, enrolled in the previous trial (treatment variant – 28 days after completion of Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration). 8 healthy rats of the similar body weight constituted a separate group. All the animals were administered intravenously with insulin (0,3 units), and glucose concentration in their blood was estimated hereafter.

The results of the study are shown in table 6. Healthy animals revealed a strong physiological decrease in glucose level, while in control animals (suffering alloxan diabetes) this index was 2,8 times lower. Alloxan diabetes animals treated with Lys-Glu-Asp-Trp-NH_2 tetrapeptide, revealed a reliable nearly two-fold decrease of the glucose level after insulin administration as compared to the control group. These data suggests Lys-Glu-Asp-Trp-NH_2 tetrapeptide ability to a great extent maintain tissue sensibility to insulin.

The properties of Lys-Glu-Asp-Trp-NH_2 tetrapeptide revealed during the study allow to recommend it for prophylactic and therapeutic application as a substance regulating blood glucose level in case of diabetes mellitus treatment.

The results of the clinical study of the proposed tetrapeptide shown below demonstrate its pharmacologic properties and confirm the possibility of the invention realization.

Example 8. Efficacy of applying Lys-Glu-Asp-Trp-NH_2 tetrapeptide in the patients with diabetes mellitus.

The investigation was carried out in 36 patients aged from 16 to 83 years (7 men, 29 women). In 23 patients there was diagnosed type 1 diabetes, in 13 patients – type 2 diabetes. The disease duration varied from 1 year to 30 years. 32 patients received insulin. The majority of the patients suffering diabetes mellitus entered the hospital decompensated. Blood glucose level in these patients on an empty stomach oscillated from 9.5 to 27 μm/l; the glycosylated haemoglobin – from 7.8 to 12.7%. All the patients were prescribed a rigid diet. All the patients were stratified randomly into 2 groups,

with respect to age, sex, duration and stage of the disease (Table 7). Alongside with standard method of treatment 16 patients were prescribed Lys-Glu-Asp-Trp-NH_2 tetrapeptide in dose of 10μg (in 1 ml of 0.9% NaCl solution) intramuscularly once a day for 10 days. 4 patients suffering type 2 diabetes mellitus were prescribed together with standard treatment course Lys-Glu-Asp-Trp-NH_2 tetrapeptide in dose of 100 μg (1 tablet) twice a day before meals during 10 days. Patients of the control group were administered with 1 ml of 0,9% of NaCl solution as a placebo following the same scheme.

The results of the study of the Lys-Glu-Asp-Trp-NH_2 tetrapeptide efficacy are shown in Table 8. In 8 patients (out of 16 treated insulin) Lys-Glu-Asp-Trp-NH_2 tetrapeptide course resulted in reduction of the insulin daily dose by 8 units on average, this allowed to achieve the compensation.

For 6 patients the dose of insulin remained unchanged and there was registered a compensation. Only for two patients in order to achieve compensation the insulin dose was increased: the 1st patient – by 4 units; the 2nd patient – by 14 units. At the same time in control group (16 insulin administered patients) only for 2 patients the dose of insulin remained unchanged, for 14 patients the dose of insulin was increased by 4-8 units in order to achieve compensation.

In patients of the main group suffering type 2diabetes mellitus (1 patient) and type 1 diabetes mellitus (3 patients), who received Lys-Glu-Asp-Trp-NH_2 tetrapeptide in tablets daily, the dose of insulin was reduced by 25 units and in patients, who were treated with oral anti-diabetic medicine, there was achieved a full compensation and the dose of preparations was reduced practically two-fold.

Thus, the application of Lys-Glu-Asp-Trp-NH_2 tetrapeptide in patients suffering diabetes mellitus contributed to the increase of tissues sensitivity towards insulin and to some extent to pancreas function restoration. It should be noticed that there was registered rather high effectiveness of Lys-Glu-Asp-Trp-NH_2 tetrapeptide, which was evidenced by the decrease of insulin dose in 50% of patients of the main group, while none of the patients in control group revealed this result.

In confirmation of the said above we adduce 3 short extracts from the case histories.

Extract from the Case history № 1.

Patient K., 69 years old, group 2 invalid. The patient has been suffering diabetes mellitus for 17 years. Since1986 she has received anti-diabetic medicine in tablets, since 1996 these drugs were substituted with insulin. The examination of the patient revealed late diabetic complications.

Diagnosis: type 2 diabetes mellitus, secondary resistance towards insulin, diabetic retinopathy, polyneuropathy, diabetic nephropathy, symptomatic hypertension.

On the admission for treatment: blood glucose level – 10 μmol/l, two hours after meals - 14μmol/l. Blood clinical analysis – normal, proteinuria - up to 0,66 g/l, ECG –hypertrophy of the left heart ventricle. Daily dose of insulin – 82 units.

Therapy: diet, vitamins of B group, berlition, parenteral form of the pharmaceutical composition, containing Lys-Glu-Asp-Trp-NH_2 tetrapeptide, 10 μg intramuscularly during 10 days.

Discharge from the hospital on the 18th day: blood glucose level on an empty stomach - 5,9 μmol/l. Daily dose of insulin – 56 units (decrease by 26 units as compared to the initial level). There is a pronounced improvement in coagulogram indices.

Extract from the Case history № 2

Patient M., 40 years, group 2 invalid. He has been suffering diabetes mellitus for 13 years and right from the onset of the disease treated with insulin.

Diagnosis: type 1 diabetes mellitus, average stage, diabetic retinopathy, polyneuropathy, encephalopathy.

On the admission for treatment: Blood glucose level on an empty stomach - 17,8 μmol/l. Clinical blood and urine analysis – normal. Daily dose of insulin - 40 units.

Therapy: diet, vitamins of B group, parenteral form of the pharmaceutical composition, containing Lys-Glu-Asp-Trp-NH_2 tetrapeptide, 10 μg intramuscularly during 10 days.

Discharge from the hospital on the 15th day: blood glucose level on an empty stomach - 3,4 μmol/l. Daily norm of insulin – 32 units (decrease by 8 units as compared to the initial level).

Extract from the Case history № 3.

Patient L., 83 years old. She has been suffering diabetes mellitus for 25 years, was treated with different antidiabetic pills. Follows strict diet, recent state of the patient is satisfactory.

Diagnosis: Type 2 diabetes mellitus, diabetic retinopathy.

On admission for treatment: blood glucose level on an empty stomach - 11 μmol/l. Clinical blood and urine analysis - normal. Takes 2 tab. of diabeton daily. As the patient had revealed resistance towards pilled antidiabetic preparations she was recommended to take insulin. But the patient declined to be treated with insulin, that was why she was prescribed oral form of pharmaceutical composition, containing Lys-Glu-Asp-Trp-NH_2 tetrapeptide, 100 μg (1 tab) twice a day before meals during 10 days together with the intake of 2 diabeton tablets. On the second day glucose level on an empty stomach was 6 μmol/l. Then the dose of diabeton was reduced two-fold. After the completion of the treatment course with Lys-Glu-Asp-Trp-NH_2 tetrapeptide the blood glucose level remained within the norm. Present state of the patient is satisfactory.

Table 1

Animal group	Glucose concentration in the blood (mg %)								
	Initial level	15 days after alloxan administra tion	8 days since the onset of Lys-Glu-Asp-Trp-NH_2 tetrapeptide administra tion	Upon completion of peptide Lys-Glu-Asp-Trp-NH_2 administration (days)					
				1	9	18	28	44	58
Control (NaCl)	84.0 ± 5.7	345.0 ± 15.4	360.0 ± 12.3	342.5 ± 17.3	351. 4 ± 11.2	368.3 ± 8.1	375.7 ± 11.2	347.2 ± 12.8	332.1 ± 13.7
n	10	10	9	8	7	6	5	5	3
Tetrapeptide Lys-Glu-Asp-Trp-NH_2	81.1 ± 3.8	333.6 ± 12.4	254.5 ± 16.2*	222.5 ± 10.3*	183.9 ± 10.5*	236.7 ± 10.3*	221.5 ± 11.2*	210.8 ± 9.3*	198.9 ± 11.5*
n	11	11	11	10	9	9	8	8	7

* - P<0.001 as compared to the control.

Table 2

Animal group	Glucose concentration in blood (mg %)							
	Initial level	7 days from the onset of Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration (1 course)	After alloxan administration (days)					
			5	14	21	28	33	40
					2nd course of Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration (from 18th till 28th day)			
Control (NaCl)	82.7 ± 0.9	96.4 ± 1.0	351.7 ± 19.4*	333.7 ± 55.8*	345.6 ± 57.8*	156.4 ± 26.4*	383.0 ± 89.3*	405.0 ± 89.8*
n	7	7	7	6	5	5	4	4
Tetrapeptide Lys-Glu-Asp-Trp-NH_2	76.8 ± 1.1	94.0 ± 0.8	247.3 ± 17.2*#	261.5 ± 39.5*#	159.0 ± 32.6*#	77.3 ± 1.3#	90.7 ± 5.2#	107.7 ± 6.4#
n	8	8	8	8	6	6	6	6

* - $P<0.001$ as compared to the control;

- $P<0.02$ as compared to the control.

Table 3

Animal group	Glucose concentration in blood (mg %)							
	Initial level	After alloxan administration (days)		After Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration (days)				
		7	14	1	4	7	14	21
Control (NaCl)	78.6 ± 4.2	276.4 ± 0.9	272.1 ± 9.8	275.7 ± 9.7	278.6 ± 9.8	277.9 ± 11.1	275.0 ± 10.5	285.0 ± 12.9
n	7	7	7	7	7	7	6	6
Tetrapeptide Lys-Glu-Asp-Trp-NH_2 (1 mkg)	80.6 ± 2.8	245.6 ± 12.3	261.7 ± 12.3	228.3 ± 10.7*	244.4 ± 12.2*	269.4 ± 8.6	268.8 ± 12.4	272.5 ± 9.9
n	9	9	9	9	8	8	8	8
Tetrapeptide Lys-Glu-Asp-Trp-NH_2 (10 mkg)	83.9 ± 3.4	247.2 ± 14.0	252.2 ± 7.9	193.3 ± 6.7*	212.2 ± 7.3*	205.6 ± 8.4*	240.0 ± 7.9*	248.3 ± 13.5
n	7	7	7	7	7	7	6	6

* - $P<0.05$ as compared to the control.

Table 4

Animal group	Insulin content in blood (mkIU/ml)							
	Initial level	15 days after alloxan administration	8 days since the onset of Lys-Glu-Asp-Trp-NH_2 tetrapeptide administration	After the completion of tetrapeptide Lys-Glu-Asp-Trp-NH_2 administration (days)				
				1	9	18	28	44
Control (NaCl)	24.3 ± 2.1	2.0 ± 0.7	0.8 ± 0.25	0	0	0	0	0
n	8	8	8	7	6	5	5	5
Tetrapeptide Lys-Glu-Asp-Trp-NH_2	23.8 ± 2.8	1.5 ± 0.4	3.1 ± 1.1*	3.6 ± 0.7#	3.2 ± 0.5#	4.3 ± 0.5#	4.1 ± 0.6#	3.9 ± 1.1#
n	10	10	10	8	7	7	7	7

* - $P<0.05$ as compared to the control;
# - $P<0.001$ as compared to the control.

Table 5

Animal group	Initial level	Time after glucose administration (min)			
		5	30	60	120
Healthy	84.5 ± 4.2	172.3 ± 8.1	132.1 ± 9.1	105.3 ± 6.2	96.8 ± 5.3
n	7	7	7	7	7
Control (NaCl)	347.2 ± 12.8*	450.5 ± 15.2*	442.7 ± 14.3*	428.9 ± 14.1*	420.5 ± 16.5*
n	5	5	5	5	5
Tetrapeptide Lys-Glu-Asp-Trp-NH_2	210.8 ± 9.3#	301.2 ± 10.5#	205.1 ± 11.8#	201.5 ± 13.2#	164.2 ± 12.8#
n	8	8	8	8	8

* - $P<0.001$ as compared to healthy animals;
# - $P<0.05$ as compared to the control.

Table 6

Animal group	Glucose concentration in blood (mg %)		Decrease in glucose content with respect to initial level (%)
	Initial level	30 minutes after insulin administration	
Healthy	83.5 ± 3.2	29.4 ± 2.2	64.8
n	8	8	
Control (NaCl)	375.7 ± 11.2*	287.8 ± 12.5*	23.4
n	5	5	
Tetrapeptide Lys-Glu-Asp-Trp-NH_2	221.5 ± 11.2#	123.6 ± 8.3#	44.2
n	8	8	

* - P<0.001 as compared to healthy animals;
# - P<0.05 as compared to the control.

Table 7

Index	Group of patients	
	Control (placebo)	Main (tetrapeptide Lys-Glu-Asp-Trp-NH_2)
Number of patients	16	20
Men	3	4
Women	13	16
Number of patients with type 1 diabetes mellitus	11	12
Number of patients with type 2 diabetes mellitus	5	8

Table 8

Index	Group of patients	
	Control (placebo)	Main (tetrapeptide Lys-Glu-Asp-Trp-NH_2)
Number of patients	16	16
Decreased insulin dose necessary to achieve compensation	0	8
The same insulin dose necessary to achieve compensation	2	6
Increased insulin dose necessary to achieve compensation	14	2